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Quantitation of endogenously occupied and unoccupied binding sites for 1,25-dihydroxyvitamin D_3 in rat intestine

(receptors/vitamin D/intestinal calcium transport)

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ABSTRACT **The quantitative reversible dissociation of the 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]-receptor complex by the mercurial reagent mersalyl was used to develop an assay for endogenously occupied and unoccupied 1,25-$(OH)_2D_3$ binding sites. Incubation of intestinal cytosol preparations in buffer containing 50 mM Tris·HCl, 300 mM KCl, and 1.5 mM EDTA, pH 7.4, with 1 mM mersalyl for 60 min was effective in inhibiting 98% of 1,25-$(OH)_2D_3$ specific binding activity. Dissociation of bound 1,25-$(OH)_2$[26,27-^{3}H]D_3 from the hormone–receptor complex approached completion by 180 min. In cytosol incubated with saturating levels of nonradioactive hormone, 96% of total binding activity was measurable with the hormone binding assay after displacement of bound nonradioactive ligand with 1 mM mersalyl. Endogenously occupied 1,25-$(OH)_2D_3$ binding sites contributed 0, 9, and 19% of total binding activity in rats with plasma 1,25-$(OH)_2D_3$ levels averaging 2, 121 ± 36 and 516 ± 92 pg/ml, respectively. Therefore, the major fraction of cytosolic 1,25-$(OH)_2D_3$ specific binding activity is unoccupied in rat intestine. The results suggest that only a small proportion of the measurable receptors are in the bound form to provide maximal 1,25-$(OH)_2D_3$-induced calcium transport.**

Association of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the hormonally active form of vitamin D_3, with a specific cytosolic binding protein, or receptor, is considered to be an obligatory initial event for expression of 1,25-$(OH)_2D_3$ action on intestinal calcium transport (1, 2). For several steroid hormones, reactive sulfhydryl groups of the receptor are essential to its ability to associate with hormone (3–5). Several lines of evidence suggest that cysteine residues similarly participate in the binding of 1,25-$(OH)_2D_3$ to its receptor. Studies by Kream and co-workers (6) in this laboratory showed a stabilization of the cytosolic 1,25-$(OH)_2D_3$ binding protein in the presence of excess thiol reagent. Additionally, both alkylating reagents (7, 8) and organic mercurial compounds (8) are effective inhibitors of 1,25-$(OH)_2D_3$ specific binding activity. More importantly, Coty (8) has recently demonstrated the reversible dissociation of the 1,25-$(OH)_2D_3$–receptor complex in chick intestine by sulfhydryl-blocking mercurial reagents. This observation was extended by Pike (9), who observed a dissociation of the 1,25-$(OH)_2D_3$ binding protein from DNA-cellulose by mercurial reagents. In the present studies we have investigated the use of the mercurial reagent mersalyl in developing an assay for endogenously occupied and unoccupied 1,25-$(OH)_2D_3$ binding sites in rat intestine.

Considerable evidence suggests that target cell responsiveness depends on receptor concentration (10, 11). Regulation of receptor concentration in target cells by the homologous hormone is well established for both peptide (10) and steroid (11) hormones. Furthermore, regulation of 1,25-$(OH)_2D_3$ receptor concentration by exogenous glucocorticoids has recently been demonstrated (12, 13). It is clear that the cytosolic 1,25-$(OH)_2D_3$ specific binding protein may exist in a free, unoccupied state or bound to endogenous ligand. Most studies, however, have only examined unoccupied 1,25-$(OH)_2D_3$ receptor sites. Therefore, the relationship between 1,25-$(OH)_2D_3$ receptor concentration and the response of intestinal calcium transport to circulating 1,25-$(OH)_2D_3$ levels in the rat remains to be determined. To gain information on the influence of circulating 1,25-$(OH)_2D_3$ on its own receptor sites, we utilized the reversible dissociation of 1,25-$(OH)_2D_3$-specific binding activity by mersalyl to quantitate endogenously occupied and unoccupied 1,25-$(OH)_2D_3$ receptor levels in rat intestine. Mersalyl was effective in inhibiting the binding of 1,25-$(OH)_2D_3$ and in displacing bound 1,25-$(OH)_2D_3$ from the hormone–receptor complex as previously reported for the analogous cytosolic receptor in chick intestine (8). The results from this assay indicate that rat intestine contains a large population of unoccupied receptors, suggesting that only a small fraction of measurable binding activity is required for 1,25-$(OH)_2D_3$-induced calcium transport.

MATERIALS AND METHODS

Chemicals. Nonradioactive 1,25-$(OH)_2D_3$ was a gift from M. Uskokovic of Hoffmann–La Roche. Purity and concentration were determined by ultraviolet absorption, using an extinction coefficient of 18,200 M^{-1} cm^{-1} at 264 nm. 1,25-$(OH)_2$[26,27-^{3}H]D_3 was synthesized as described (14) and repurified to greater than 95% purity by high-performance liquid chromatography.

Animals. Female Holtzman rats (Holtzman, Madison, WI) were obtained on day 3 of gestation and fed a vitamin D-deficient semipurified diet containing 0.47% calcium and 0.30% phosphorus throughout gestation and lactation (15). Male pups were naturally weaned to the dam's diet. Plasma 1,25-$(OH)_2D_3$ was nondetectable in pups 28 days post partum, confirming vitamin D deficiency (16). Male Holtzman rats were obtained at 21 days post partum and fed a vitamin D-supplemented diet containing adequate calcium and phosphorus as described above or a low-calcium diet containing 0.02% calcium and 0.30% phosphorus. Both groups of vitamin D-replete pups received 25 international units of vitamin D_2 per day in 0.1 ml of cottonseed/soybean oil (Wesson).

One-day-old White Leghorn cockerels (Northern Hatcheries, Beaver Dam, WI) were fed a vitamin D-deficient soy protein diet for 6–10 weeks (17).

Preparation of Cytosol. At day 28 post partum, rats were anesthetized with ether and bled from the jugular vein. Then

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Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; TKE buffer, 50 mM Tris·HCl/300 mM KCl/1.5 mM EDTA, pH 7.4; TKED buffer, TKE buffer with 5 mM dithiothreitol.

12% of the small intestine immediately adjacent to the pyloric sphincter was rapidly excised and rinsed with cold buffer containing 50 mM Tris·HCl, 300 mM KCl, 1.5 mM EDTA, and 5 mM dithiothreitol, pH 7.4 (TKED buffer). The mucosa was scraped from the serosa and washed three times in 10 vol of TKED buffer to eliminate contamination from the 6S 1,25-$(OH)_2[^3H]D_3$-binding component (18). The mucosa was homogenized in TKED buffer (30%, vol/vol) with a Teflon pestle. Cytosol was prepared by centrifugation at 90,000 × *g* for 90 min at 0–4°C in a Beckman L5-50 ultracentrifuge with a 50 Ti rotor (Beckman). Chicken intestinal cytosol was prepared in buffer containing 0.3 M KCl as described (16). Hormone binding was measured in cytosol preparations described above or in cytosol fractions brought to 40% saturation with $(NH_4)_2SO_4$. After centrifugation for 10 min at 4,000 × *g*, the resulting pellets were suspended in buffer containing 50 mM Tris·HCl, 300 mM KCl, and 1.5 mM EDTA, pH 7.4 (TKE buffer), and used in hormone binding assays. Protein concentration of cytosol preparations was determined by the Coomassie blue dye method of Bradford (19) with crystalline bovine serum albumin as standard.

Sucrose Density Gradient Analysis. Aliquots of cytosol (0.24 mg of protein) in TKE buffer were incubated with 1 nM 1,25-$(OH)_2[26,27\text{-}^3H]D_3$ in the presence or absence of 100-fold excess nonradioactive 1,25-$(OH)_2D_3$ for 3 hr at 0–4°C. To measure dissociation of bound ligand from the 1,25-$(OH)_2D_3$ binding protein, mersalyl was added after hormone binding to a final concentration of 1 mM. After 3 hr, the displacement reaction was stopped by addition of 25 mM dithiothreitol. Reassociation of hormone binding was determined after incubation with 1,25-$(OH)_2[26,27\text{-}^3H]D_3$ for 16 hr at 0–4°C. Unbound ligand was removed by addition of dextran-coated charcoal. Aliquots of charcoal-treated supernatants were analyzed on linear 4–20% sucrose density gradients as described (6). For determination of radioactivity, 3.5 ml of Scint-A (Packard, Downers Grove, IL) plus 200 μl of water for clarification were added to 0.1-ml fractions. Ovalbumin (3.7S) and bovine serum albumin (4.4S) were used as sedimentation coefficient standards.

Hormone Binding Assay. Aliquots of cytosol (0.5–1.0 mg of protein) were incubated with 1 nM 1,25-$(OH)_2[^3H]D_3$ for 3 hr at 0–4°C. Nonspecific binding was determined in parallel incubation mixtures containing 100-fold excess nonradioactive 1,25-$(OH)_2D_3$. To determine the effect of mersalyl on inhibiting 1,25-$(OH)_2D_3$ specific binding activity, cytosol preparations were incubated with mersalyl before measurement of hormone binding. Mersalyl was added to cytosol preparations at the end of the 3-hr incubation with 1,25-$(OH)_2[^3H]D_3$ to determine displacement of bound hormone. Hormone binding was measured by adsorption to hydroxylapatite (20). Washed hydroxylapatite pellets were extracted twice with 2 ml of methanol/chloroform, 2:1 (vol/vol). To determine radioactivity, the extracts were evaporated to dryness and 4 ml of scintillator (3a70B, Research Products, Elk Grove, IL) was added. Radioactivity was measured by using a PRIAS liquid scintillation counter (Packard) with a counting efficiency for tritium of 26%.

Determination of Unoccupied and Occupied Binding Sites. Unoccupied 1,25-$(OH)_2D_3$ binding sites were determined by measuring hormone binding in cytosol preparations incubated with 1 nM 1,25-$(OH)_2[26,27\text{-}^3H]D_3$ for 3 hr at 0–4°C. To determine total 1,25-$(OH)_2D_3$ specific binding activity, endogenously bound hormone was first displaced by incubation of cytosol preparations in TKE buffer with mersalyl at a final concentration of 1 mM. After various times, mersalyl was inactivated by the addition of 25 mM dithiothreitol. Total hormone binding was assayed after incubation with radioactive ligand for 16 hr at 0–4°C. Occupied binding sites were calculated as the difference between total and unoccupied binding sites.

RESULTS

The effect of the mercurial reagent mersalyl in inhibiting 1,25-$(OH)_2D_3$ specific binding activity and in displacing bound 1,25-$(OH)_2D_3$ from the hormone–receptor complex was compared in cytosol preparations containing various amounts of thiol reagent. Cytosols normally prepared in the presence of 5 mM dithiothreitol to maintain the integrity of reduced sulfhydryl groups of the 1,25-$(OH)_2D_3$ binding protein were treated with 6 mM mersalyl. This excess mersalyl in the presence of dithiothreitol was effective in inhibiting only 60% of total binding activity and in displacing 33% of bound 1,25-$(OH)_2[^3H]D_3$. Therefore, prior to assaying for hormone binding activity, cytosol preparations were brought to 40% saturation with $(NH_4)_2SO_4$ and precipitates were resuspended in buffer containing no dithiothreitol. Treatment of the cytosol preparations in TKE buffer with 1 mM mersalyl for 60 min at 0–4°C resulted in a 98% inhibition of 1,25-$(OH)_2D_3$ specific binding activity and a 76% displacement of bound 1,25-$(OH)_2D_3$.

The effect of mersalyl on the rate of release of 1,25-$(OH)_2D_3$ previously incorporated into rat intestinal cytosol is shown in Fig. 1. Cytosol preparations were incubated with 1 nM 1,25-$(OH)_2[26,27\text{-}^3H]D_3$ in the presence or absence of excess nonradioactive 1,25-$(OH)_2D_3$ at 0–4°C. The displacement reaction was initiated after 3 hr by the addition of mersalyl to a final concentration of 1 mM. Control binding activity in the absence of dithiothreitol did not significantly decrease throughout this time course, averaging 408 ± 30 fmol/mg of cytosolic protein (mean ± SD). The displacement reaction approached completion by 180 min, with 6 ± 3% of control 1,25-$(OH)_2D_3$ specific binding activity remaining. The recovery of binding activity after reversing the mersalyl displacement reaction with excess dithiothreitol and allowing rebinding to occur overnight at 0–4°C routinely averaged greater than 70%. As shown in Fig. 2, the rate of displacement of binding activity appeared to be log-linear, with a $t_{1/2}$ of approximately 40 min. The rate of displacement of bound hormone from the 1,25-$(OH)_2D_3$ binding protein was considerably faster in chick intestinal cytosol preparations, with a $t_{1/2}$ of 5 min (Fig. 2). This is in agreement with

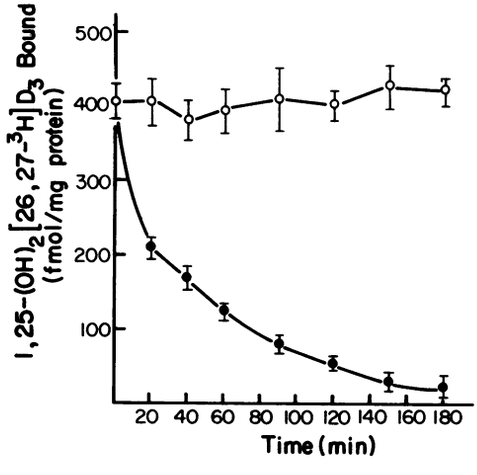

FIG. 1. Rate of dissociation of bound 1,25-$(OH)_2[26,27\text{-}^3H]D_3$ in rat intestinal cytosol treated with mersalyl. Cytosol in TKE buffer was incubated with 1 nM 1,25-$(OH)_2[26,27\text{-}^3H]D_3$ in the presence or absence of 100-fold excess nonradioactive 1,25-$(OH)_2D_3$. After 3 hr at 0–4°C, dissociation of the hormone–receptor complex was initiated (0 time) by addition of 1 mM mersalyl. At various time points up to 180 min, mersalyl was inactivated by the addition of 25 mM dithiothreitol (●). Control binding in the absence of mersalyl was measured throughout the incubation period (○). Results are mean ± SD for triplicate determinations.

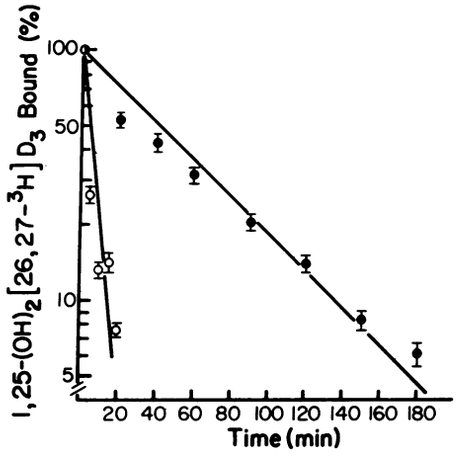

FIG. 2. Displacement of bound 1,25-$(OH)_2$[26,27-^{3}H]D_3 by mersalyl treatment of rat (●) and chick (○) intestinal cytosol. Dissociation of bound 1,25-$(OH)_2$[^{3}H]D_3 in intestinal cytosol preparations was measured as described in the legend to Fig. 1.

a $t_{1/2}$ of 7 min previously reported by Coty (8) for chick intestine. The difference in the ability of mersalyl to displace bound 1,25-$(OH)_2D_3$ may indicate a slower rate of reaction of this mercurial reagent with sulfhydryl groups of the mammalian 1,25-$(OH)_2D_3$ cytosolic binding protein.

The regeneration of 1,25-$(OH)_2D_3$ specific binding after displacement of bound hormone in the presence of 1 mM mersalyl was examined by sucrose density sedimentation. Preparations of control cytosol exhibited binding activity sedimenting in the 3.2–3.7S region characteristic of the cytosolic receptor for 1,25-$(OH)_2D_3$ in rat intestine (6, 7, 21). This peak was completely displaced when cytosol was incubated in the presence of excess nonradioactive 1,25-$(OH)_2D_3$ (Fig. 3*A*). As shown in Fig. 3*B*, mersalyl effectively dissociated bound 1,25-$(OH)_2$[^{3}H]D_3 from the hormone–receptor complex. When hormone binding was allowed to occur in the presence of excess dithiothreitol, a single peak containing 1,25-$(OH)_2$[^{3}H]D_3 specific binding activity was regenerated (Fig. 3*C*).

With this assay used to quantitate endogenously occupied receptor sites, it was possible that nonradioactive ligand displaced from the hormone–receptor complex could compete with the radioactive 1,25-$(OH)_2D_3$ during overnight rebinding. Therefore, we next assessed the ability of this assay to quantitate receptor levels in cytosol 100% occupied with nonradioactive 1,25-$(OH)_2D_3$. Cytosol was incubated with 1 nM nonradioactive 1,25-$(OH)_2D_3$ for 16 hr at 0–4°C. Unbound hormone was separated by adsorption of the hormone–receptor complex to hydroxylapatite as described by Wecksler and Norman (20). After centrifugation, the pellet was washed twice with 50 mM Tris·HCl/1.5 mM EDTA/5 mM dithiothreitol (TED) buffer, pH 7.4, containing 0.5% Triton X-100 (20). The 1,25-$(OH)_2D_3$ binding protein was eluted from the hydroxylapatite with two washes of 50 mM Tris·HCl/1.5 mM EDTA/0.25 M KH_2PO_4, pH 7.4 (22). The eluates were concentrated by addition of saturated $(NH_4)_2SO_4$ and the resulting precipitate was suspended in 50 mM Tris·HCl/1.5 mM EDTA, pH 7.4. Quantitation of 1,25-$(OH)_2D_3$ specific binding activity in this cytosol preparation preincubated with nonradioactive hormone was compared to that of control cytosol. As shown in Fig. 4, minimal specific binding activity was detected by the hormone binding assay in cytosol preparations preincubated with saturating levels of nonradioactive 1,25-$(OH)_2D_3$ (less than 10 fmol per incubation). However, after dissociation of bound nonradioactive ligand with mersalyl and rebinding in the presence of 1 nM 1,25-$(OH)_2$-[^{3}H]D_3, greater than 96% of control binding activity was recovered.

The use of this assay in measuring endogenously occupied and unoccupied receptor sites in rat duodenum was investigated. That is, mersalyl was used to dissociate endogenously bound hormone so that total 1,25-$(OH)_2D_3$ binding sites could be measured by the hormone binding assay using radioactive ligand. Intestinal 1,25-$(OH)_2D_3$ specific binding activity was examined in cytosol preparations from rats fed diets varying in vitamin D and calcium content to obtain differences in plasma 1,25-$(OH)_2D_3$ levels between groups. The relationship between plasma 1,25-$(OH)_2D_3$ levels, total 1,25-$(OH)_2D_3$ binding activity, and the proportion of binding sites occupied by endogenous hormone is shown in Fig. 5. Total 1,25-$(OH)_2D_3$ specific binding activity in vitamin D-deficient pups at 28 days post partum averaged 577 fmol/mg of cytosolic protein. This is similar to the total binding activity of 610 fmol/mg of protein in intestinal cytosols from vitamin D-deficient dams in the present study and is in agreement with our previous observation that the adult level of 1,25-$(OH)_2D_3$ specific binding activity is present in the

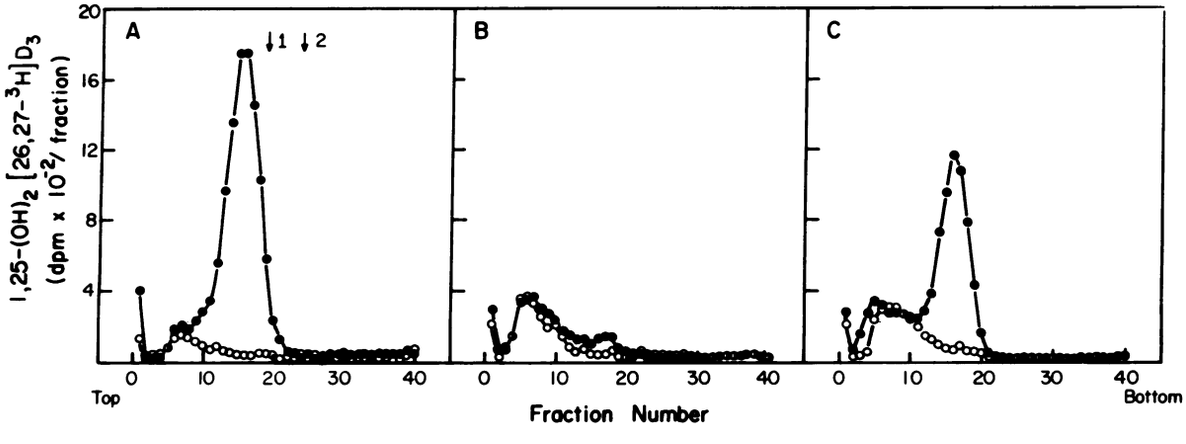

FIG. 3. Sucrose density gradient profiles of rat intestinal preparations treated with mersalyl followed by dithiothreitol and 1,25-$(OH)_2$-[26,27-^{3}H]D_3. Cytosol preparations were incubated with 1 nM 1,25-$(OH)_2$[26,27-^{3}H]D_3 and analyzed on linear 4–20% sucrose gradients. (*A*) Binding of 1 nM 1,25-$(OH)_2$[^{3}H]D_3 in the absence (●) and presence (○) of 100 nM nonradioactive 1,25-$(OH)_2D_3$. Arrows 1 and 2 refer to the sedimentation coefficient standards ovalbumin (3.7S) and bovine serum albumin (4.4S), respectively. (*B*) Displacement of bound 1,25-$(OH)_2$[^{3}H]D_3 in the absence (●) and presence (○) of excess nonradioactive 1,25-$(OH)_2D_3$ after incubation with 1 mM mersalyl for 180 min at 0–4°C. (*C*) Binding of 1,25-$(OH)_2$-[^{3}H]D_3 by receptor after mersalyl treatment followed by incubation with excess dithiothreitol for 16 hr at 0–4°C.

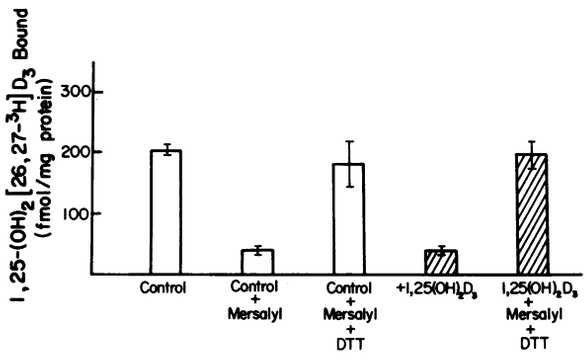

FIG. 4. Quantitation of 1,25-$(OH)_2D_3$ binding sites in cytosol preincubated with saturating levels of nonradioactive 1,25-$(OH)_2D_3$. Specific 1,25-$(OH)_2D_3$ binding activity was measured in control cytosol (open bars) or cytosol preincubated with 1 nM nonradioactive 1,25-$(OH)_2D_3$ (hatched bars) to obtain 100% occupancy of binding sites *in vitro*. DTT, dithiothreitol. Results are mean ± SD for triplicate determinations.

rat duodenum by day 28 post partum (18, 23). Total binding activity tended to be lower in cytosol preparations from rats fed diets supplemented with vitamin D when compared to the vitamin D-deficient groups. The level of endogenously occupied 1,25-$(OH)_2D_3$ binding sites was low in both groups of rats fed vitamin-D supplemented diets but increased in response to increased plasma 1,25-$(OH)_2D_3$ levels. The level of occupied binding sites was more than doubled in rats fed the vitamin D-supplemented, low-calcium diet, compared to rats fed the diet adequate in calcium. Endogenously occupied binding sites, however, did not contribute more than 19% of total 1,25-$(OH)_2D_3$ specific binding activity, suggesting that the intestine contains a large population of unoccupied receptors.

DISCUSSION

Several lines of evidence have established that reduced sulfhydryl groups of steroid hormone receptors play a role in the binding of receptor to the homologous hormone (3–8). Although direct participation of sulfhydryl groups in the hormone–receptor interaction remains to be substantiated, important characteristics of hormone binding can be determined through exposure of unpurified receptor preparations to sulfhydryl-blocking reagents. The ability of sulfhydryl-blocking reagents to inhibit binding of 1,25-$(OH)_2D_3$ to its cytosolic

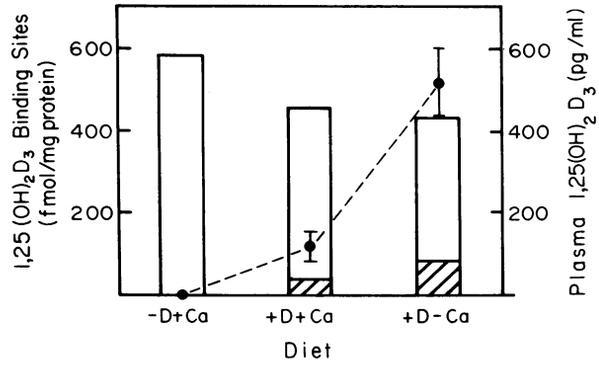

FIG. 5. Relationship between plasma 1,25-$(OH)_2D_3$ levels and 1,25-$(OH)_2D_3$ binding sites in rat intestine. D and Ca refer to vitamin D and calcium in the diet. Total (open bars) and occupied (hatched bars) 1,25-$(OH)_2D_3$ binding sites were measured in cytosol preparations as described in the text. Plasma 1,25-$(OH)_2D_3$ concentrations (●, mean ± SD) were determined according to the procedure of Eisman *et al.* (16).

receptor has been demonstrated for chick intestine (8) and for the more labile receptor from rat intestine (7). The use of sulfhydryl-blocking reagents has recently been extended by the observation of a reversible dissociation of the 1,25-$(OH)_2D_3$–receptor complex in chick intestine (8) and the glucocorticoid receptor in rat liver cytosol (24) by mercurial reagents. Additionally, the present study confirms the displacement of 1,25-$(OH)_2D_3$ specific binding activity in mammalian intestine by the mercurial reagent mersalyl.

Prior exposure to cytosol from rat intestine to mersalyl completely eliminated 1,25-$(OH)_2D_3$ specific binding activity. Mersalyl displacement of bound 1,25-$(OH)_2D_3$ from the hormone-receptor complex occurred at a slower rate in the mammalian system compared to the rate of displacement observed for chick intestine (8). This observed difference may reflect a more rapid rate of reaction of mersalyl or a greater accessibility of mersalyl to the reduced sulfhydryl groups of the cytosolic 1,25-$(OH)_2D_3$ binding protein from chick intestine.

The quantitative reversible dissociation of the 1,25-$(OH)_2D_3$–receptor complex was utilized to measure the contribution of endogenously occupied and unoccupied receptor sites to total 1,25-$(OH)_2D_3$ binding activity in rat duodenum. Our results indicate that the major proportion of 1,25-$(OH)_2D_3$ binding sites is unoccupied by endogenous ligand. Occupied binding sites contributed only 9% of total binding activity in rats fed a vitamin D-supplemented diet adequate in calcium and phosphorus. This is in agreement with the proportion of endogenously occupied binding sites in normal chick intestine measured by the exchange assay of Hunziker *et al.* (25). These data indicate that "spare receptors" that are not important for biologic response exist for 1,25-$(OH)_2D_3$ in the intestine. The data may also result from the *in vitro* handling of the occupied receptor. The techniques for receptor isolation may increase the rate of dissociation of endogenous 1,25-$(OH)_2D_3$ and result in quantitation of occupied sites as unoccupied. However, our results suggest that the level of occupied binding sites in rat duodenum is dependent on plasma 1,25-$(OH)_2D_3$ concentration. Increased plasma 1,25-$(OH)_2D_3$ levels produced in rats adapted to a vitamin D-supplemented low-calcium diet caused a 2-fold increase in occupied binding sites. Therefore, our data suggest that the responsiveness of intestinal calcium transport to 1,25-$(OH)_2D_3$ is related to the level of cytosolic receptors bound to endogenous ligand.

It is of interest that total 1,25-$(OH)_2D_3$ specific binding activity and the level of unoccupied binding sites in intestine tended to be lower in rats fed diets supplemented with vitamin D. The proportion of unoccupied binding sites in duodenum from vitamin D-replete rats was 30–40% lower than that in the vitamin D-deficient group. We have previously reported a similar decrease in rat intestinal 1,25-$(OH)_2D_3$ binding activity contributed by unoccupied binding sites in response to vitamin D supplementation during postnatal development (18). This finding may be species specific, because quantitation of chicken intestinal receptors revealed that vitamin D had no effect on total receptor levels (26). An inverse relationship between plasma 1,25-$(OH)_2D_3$ levels and total 1,25-$(OH)_2D_3$ binding activity may indicate that the total "active" receptor concentration is regulated by circulating hormone levels. Alternatively, it may be found that receptors that are occupied *in vivo* may undergo a change that does not allow quantitation of the occupied site by an *in vitro* exchange assay. A biological advantage of a decrease in total binding activity in response to increased plasma 1,25-$(OH)_2D_3$ levels remains to be elucidated.

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